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Humanization of an Antibody Directed Against IgE

Leonard G. Presta,[1]* Steven J. Lahr,* Robert L. Shields,† James P. Porter,‡ Cornelia M. Gorman,§ Brian M. Fendly,‡ and Paula M. Jardieu†

Departments of *Protein Engineering, †Immunology, ‡Medicinal and Analytical Chemistry, and §Cell Genetics, Genentech, Inc., South San Francisco, CA 94080

Abstract. IgE antibodies bind to specific high-affinity receptors on mast cells, leading to mast cell degranulation and release of mediators, such as histamine, which produce symptoms associated with allergy. Hence, anti-IgE antibodies that block binding of IgE to its high-affinity receptor are of potential therapeutic value in the treatment of allergy. These antibodies must also not bind to IgE once it is bound to the receptor because this would trigger histamine release. This study describes the humanization of a murine antibody, MaE11, with these characteristics. Variants of the humanized antibody were evaluated to probe the importance of framework residues on antibody binding and to determine which charged residues in the CDR interacted with IgE. We found that only five changes in human framework residues were required to provide for binding comparable to that of the original murine antibody. *Journal of Immunology,* 1993, 151: 2623.

IgE antibodies bind to a specific high-affinity receptor (FcεRI) (1, 2) on mast cells and basophils via their Fc region (constant domains Cε2, Cε3, and Cε4) (3), leading to mast cell degranulation and release of mediators that produce symptoms associated with allergy (4, 5). IgE also binds to a low-affinity receptor (FcεRII and CD23) (6) on B lymphocytes (7, 8) and on cells involved in inflammation, leading to IgE-mediated cytotoxicity and phagocytosis (9). Hence, anti-IgE antibodies that block binding of IgE to its receptors may be of therapeutic value.

Herein we report the humanization of a murine antibody, MaE11, directed against IgE that prevents binding of free IgE to FcεRI on mast cells but does not bind to FcεRI-bound IgE. The latter characteristic is important because this antibody will not trigger histamine release by cross-linking IgE-loaded FcεRI on mast cells. However, as a therapeutic the murine antibody would not be the molecule of choice because clinical use of non-human antibodies has identified three fundamental problems. First, non-human antibodies cause a human immune response that can reduce therapeutic value of the non-human antibody (10–14). Second, therapeutic efficacy is reduced by the relatively rapid clearance of the non-human antibody compared with human ones (15). Finally, non-human antibodies generally show only weak recruitment of effector functions (e.g., antibody-dependent cell-mediated cytolysis), which may be desirable or essential for efficacy (16, 17).

One approach to overcoming these problems involves production of "humanized" antibodies that significantly reduce the amount of non-human sequence in the molecule. This technique, pioneered by Winter et al. (16, 18), involves transplantation of the non-human Ag-binding loops (CDR)[2] onto a human antibody framework. In addition to CDRs, select non-human framework residues must also be incorporated into the humanized antibody to maintain proper CDR conformation (19) or because they interact directly with the antigen (20). The humanized antibody, when properly constructed, will contain approximately 5% non-human residues, most of which will be in the CDRs. Although several humanized antibodies have been reported (21–28), only recently has the clinical efficacy of these

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[1] Address correspondence and reprint requests to Dr. Leonard Presta, Department of Protein Engineering, Genentech, Inc., 460 Point San Bruno Blvd., South San Francisco, CA 94080.

[2] Abbreviations used in this paper: CDR, complementarity-determining region.

EXHIBIT "B"

molecules begun to be evaluated (15).

Most humanized antibodies have been designed by comparing the sequence of the murine antibody of interest to a database of human antibody sequences and choosing the human antibody most homologous to that of the murine antibody (23–27). In contrast, we have used a framework derived from consensus sequences of human VL and VH subgroups. This provides for use of the most common framework found among human IgG antibodies and eliminates possible idiosyncracies present in any individual framework, both of which would, hopefully, reduce the chance of an immunogenic response against the humanized antibody. This same framework has been previously used for other humanized antibodies (21, 22).

Thirteen variants of the humanized antibody were evaluated to probe the importance of framework residues on antibody binding. We found that only five changes in human framework residues were required to provide for binding comparable to that of the original murine antibody. We also used an additional 10 variants to ascertain whether any of the charged CDR residues were important IgE-binding determinants.

Materials and Methods

Construction of humanized antibody

The murine anti-human IgE mAb, MaE11, was generated, cloned, and sequenced at Genentech (unpublished data). To construct the first F(ab) variant of humanized MaE11, F(ab)-1, site-directed mutagenesis (29) was performed on a deoxyuridine-containing template containing a human κ-subgroup I light chain and human subgroup III heavy chain (VH-CH1) in a pUC119-based plasmid, pAK2 (21). F(ab)-2 was then constructed from a F(ab)-1 template. All other humanized F(ab) variants were constructed from a template of F(ab)-2. Plasmids were transformed into *Escherichia coli* strain JM101 (30) for preparation of double- and single-stranded DNA. For each variant both light and heavy chains were completely sequenced using the dideoxynucleotide method. DNA encoding light and heavy chains was then subcloned into a derivative of the *E. coli* F(ab) expression plasmid, pAK19 (31). These derivatives lack the hinge cysteines that form the interheavy chain disulfides in $F(ab')_2$ fragments. F(ab) fragments, as opposed to full-length IgG antibodies, facilitated the analysis of a moderately large number of variants because *E. coli* expression could be used rather than mammalian cell culture techniques. Once the best variant was determined, it was subsequently subcloned into a plasmid encoding a full-length human IgG1 (see below).

The expression plasmids were transformed into *E. coli* strain MM294 (32), and a single colony was grown in 5-ml 2YT-100 μg/ml carbenicillin for 5–8 h at 37°C. The 5-ml and allowed to grow for 16 h in a 500-ml shaker flask at 37°C. The culture was centrifuged at 4,000 × *g* and the supernatant removed. After freezing for 1 h, the pellet was resuspended in 5-ml cold 10 mM Tris-1 mM EDTA; 50 μl 0.1 M benzamidine (Sigma, St. Louis) was added to inhibit proteolysis. After gentle shaking on ice for 1 h, the sample was centrifuged at 10,000 × *g* for 15 min. The supernatant was applied to a protein A-Sepharose CL-4B (Pharmacia) column (0.5 ml bed volume) then washed with 10 ml 3 M KCl-100 mM Tris, pH 8.0, and eluted with 2.5 ml 100 mM acetic acid, pH 2.8, into 0.5 ml 1 M Tris, pH 8.0. The F(ab) was then buffer exchanged into PBS using a Centricon-30 (Amicon) and concentrated to a final volume of 0.5 ml. SDS-PAGE gels of all F(ab)s were run to ascertain purity. F(ab) concentrations were determined using an 0.1% ϵ_{280} of 1.0. The extinction coefficient was determined by using the concentration of protein from an amino acid analysis of purified F(ab)-2 and the A_{280} for this same sample.

Selected F(ab) fragments were analyzed directly by liquid chromatography/mass spectrometry to confirm their molecular weight. Samples were injected into a packed capillary liquid chromatography system (33) and analyzed directly with a Sciex API 3 mass spectrometer. The higher charge states of human growth hormone (m.w. = 22,256.2), acquired using the same instrument parameters as those used for the samples, were used for calibration.

For generation of human IgG1 versions of humanized MaE11, the heavy and light chains were subcloned separately into previously described pRK plasmids (34). Appropriate heavy and light chain plasmids were cotransfected into an adenovirus-transformed human embryonic kidney cell line, 293 (35), using a high efficiency procedure (35, 36). Media was changed to serum free and harvested daily for up to 5 days. Antibodies were purified from the pooled supernatants using protein A-Sepharose CL-4B (Pharmacia). The eluted antibody was buffer exchanged into PBS by G25 gel filtration, concentrated by ultrafiltration using a Centriprep-30 or Centricon-100 (Amicon), sterile filtered using a Millex-GV (Millipore), and stored at 4°C. The concentration of antibody was determined using total Ig-binding ELISA. The concentration of the standard was determined by amino acid composition analysis.

Soluble receptor assay

A 96-well assay plate (Nunc) was coated with 0.05 ml 1 μg/ml FcεRI α-chain IgG chimeric receptor (Genentech; unpublished data) in coating buffer (50 mM carbonate/bicarbonate, pH 9.6) for 12 h at 4–8°C. The wells were aspirated and 250 μl blocking buffer (PBS, 1% BSA, pH 7.2) was added and incubated for 1 h at 4°C. In a separate assay plate the samples and reference murine MaE11 were titered from 200 to 0.001 μg/ml by 1:4 dilutions with assay

buffer (0.5% BSA and 0.05% Tween 20, PBS, pH 7.2) and an equal volume of 10 ng/ml biotinylated IgE (37) was added and the plate incubated for 2–3 h at 25°C. The FcεRI-coated wells were washed three times with PBS and 0.05% Tween 20 (Sigma) and 50 μl from the sample wells were transferred and incubated with agitation for 30 min at 25°C. Fifty μl/well of 500 μg/ml Streptavidin-HRP (Sigma), diluted 1:5000 in assay buffer, was incubated for 15 min with agitation and then the plate was washed as before. Fifty μl/well of Microwell Peroxidase Substrate (Kirkgaard & Perry Laboratories) was added and color was developed for 30 min. The reaction was stopped by adding an equal volume of 1 N HCl, and the absorbance measured at 450 nm. The concentration at 50% inhibition was calculated by plotting percentage of inhibition versus concentration of blocking antibody with a nonlinear four-parameter curve fit using the Kaleidagraph data analysis application (Synergy Software).

FACS-based binding assays

The ability of the antibody to inhibit FITC-conjugated (38) IgE binding to the α-chain of the high-affinity FcεRI receptor expressed on CHO 3D10 cells (39) was determined by flow cytometry. FITC-conjugated IgE (40 nM) was preincubated with the antibody ($0.3–1 \times 10^{-6}$ M) at 37°C for 30 min in FACS buffer (PBS, 0.1% BSA, and 10 mM sodium azide, pH 7.4). The complex was then incubated with 5×10^5 CHO 3D10 cells at 4°C for 30 min. The cells were washed three times with FACS buffer and mean channel fluorescence at 475 nm measured on an FACScan flow cytometer (Becton Dickinson). MaE1 (Genentech), a murine anti-human IgE mAb that does not block IgE binding to the FcεRI α-chain, was used as a positive control and MOPC21 (Cappel), a murine monoclonal that does not recognize IgE, was used as a negative control.

Binding of antibodies to IgE-loaded FcεRI

Antibody binding to human IgE associated with the α-subunit of FcεRI expressed on CHO 3D10 cells (39) was determined by preincubating 5×10^5 CHO 3D10 cells with 10 μg/ml human IgE for 30 min at 4°C. Cells were washed three times followed by a 30-min incubation with varying concentrations of either murine anti-human IgE mAbs MaE1 or MaE11 or the humanized mAb variant 12. MOPC21 (murine IgG1) was used as a control for the murine mAbs, whereas humanized 4D5 mAb (21) (human IgG1) was used as a control for humanized variant 12. Binding of murine mAbs was detected with a FITC-conjugated $F(ab')_2$ goat anti-mouse IgG (10 μg/ml). Humanized mAb binding was detected with a FITC-conjugated $F(ab')_2$ goat anti-human IgG (50 μg/ml), which had been affinity purified on an IgE column to minimize cross-reactivity to IgE.

VH domain

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MaE11     DVQLQESGPGLVKPSQSLSLACSVTGYSITS[GYSWN]WIRQF
F(ab)-2   EVQLVESGGGLVQPGGSLRLSCAVSGYSITS[GYSWN]WIRQA
humIII    EVQLVESGGGLVQPGGSLRLSCAASGFTF-S[DYAMS]WVRQA
          1        10        20        30        40

MaE11     PGNKLEWMG[SITYDGSSNYNPSLKN]RISVTRDTSQNQFFL
F(ab)-2   PGKGLEWVA[SITYDGSTNYADSVKG]RFTISRDDSKNTFYL
humIII    PGKGLEWVA[VISNGSDTYYADSVKG]RFTISRDDSKNTLYL
                   50        60        70        80

MaE11     KLNSATAEDTATYYCAR[GSHYFGHWHFAV]WGAGTTVTVSS
F(ab)-2   QMNSLRAEDTAVYYCAR[GSHYFGHWHFAV]WGQGTLVTVSS
humIII    QMNSLRAEDTAVYYCAR[DSRFF-----DV]WGQGTLVTVSS
            abc       90        100abcd           110
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VL domain

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MaE11     DIQLTQSPASLAVSLGQRATISC[KASQSVDYDGDSYMN]WYQQKP
F(ab)-2   DIQLTQSPSSLSASVGDRVTITC[RASQSVDYDGDSYMN]WYQQKP
humκI     DIQMTQSPSSLSASVGDRVTITC[RASQSVDIS--SYLN]WYQQKP
          1        10        20        30  abcd      40

MaE11     GQPPILLIY[AASYLGS]EIPARFSGSGSGTDFTLNIHPVEE
F(ab)-2   GKAPKLLIY[AASYLES]GVPSRFSGSGSGTDFTLTISSLQP
humκI     GKAPKLLIY[AASSLES]GVPSRFSGSGSGTDFTLTISSLQP
                   50        60        70        80

MaE11     EDAATFYC[QQSHEDPYT]FGAGTKLEIK
F(ab)-2   EDFATYYC[QQSHEDPYT]FGQGTKVEIK
humκI     EDFATYYC[QQYNSLPYT]FGQGTKVEIK
                   90        100
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FIGURE 1. Amino acid sequences of murine MaE11, humanized MaE11 variant 2 [F(ab)-2], and human consensus sequences of heavy chain subgroup III (humIII) and light chain κ subgroup I (humκI) (42). Murine residues are italicized. The definition of CDR residues of Kabat et al. (42) are included within brackets; the definition by Chothia et al. (19) are in boldface. Kabat et al. (42) numbering used with insertions shown as a, b, c.

Computer graphics models of murine and humanized F(ab)s

Sequences of the VL and VH domains (Fig. 1) were used to construct a computer graphics model of the murine MaE11 VL-VH domains; this model was used to determine which framework residues should be incorporated into the humanized antibody. Models of the humanized variants were also constructed to verify correct selection of murine framework residues. Construction of the models was performed as described previously (21, 40).

Results

Expression and purification of humanized MaE11 F(ab)s and antibodies

Shaker flask expression levels of F(ab)s were usually 0.5–1 mg/L of culture. Full-length antibody recoveries ranged

Table I
Humanized MaE11 F(ab) variants

Variant	Changes from F(ab)-2[a]		Purpose	Concentration at 50% inh. (ng/ml)		F(ab)-X	F(ab)-X
	VL	VH		Mean	SD[b]	F(ab)-2	MaE11
F(ab)-1	*Leu* 4 Met Arg 24 *Lys* Glu 55 *Gly* Gly 57 *Glu*	*Val* 24 Ala *Ile* 37 Val Thr 57 *Ser* Ala 60 *Asn* Asp 61 *Pro* Val 63 *Leu* Gly 65 *Asn* *Phe* 78 Leu Leu 78 *Phe*	Straight CDR swap	>100,000		>16.0[c]	>560
F(ab)-1B[d]				98,000		16.0	547
F(ab)-2				6083	1279	1.0	34
F(ab)-3	*Leu* 4 Met *Met* 33 Leu		Packing; CDR-L1	9439	508	1.6	53
F(ab)-4	*Leu* 4 Met		Packing; CDR-L1	6770	349	1.1	38
F(ab)-5		*Val* 24 Ala	Packing; CDR-H1	9387	733	1.6	52
F(ab)-6		*Phe* 78 Leu	Packing; CDR-H1, H2	17,537	4372	2.9	24
F(ab)-7		*Ile* 37 Val	VL-VH interface	8622	107	1.4	48
F(ab)-8	Glu 55 *Gly* Gly 57 *Glu*		Unusual Gly 55-X-Glu 57 MaE11 sequence	5799	523	1.0	32
F(ab)-9		Ala 60 *Asn* Asp 61 *Pro*	CDR-H2; Ala 60 *Asn* at VL-VH interface	1224	102	0.20	6.8
F(ab)-10	Ala 13 *Val* Val 19 *Ala* Val 58 *Ile* Leu 78 *Val* Val 104 *Leu*	Val 48 *Met* Ala 49 *Gly* Ala 60 *Asn* Val 63 *Leu* Phe 67 *Ile* Ile 69 *Val* Met 82 *Leu* Leu 82c *Ala*	Repack F(ab)-2 interior as in murine MaE11	842	130	0.14	4.7
F(ab)-11		Ala 60 *Asn* Asp 61 *Pro* Val 63 *Leu* Phe 67 *Ile*	CDR-H2; packing of *Leu* 63 and *Ile* 67	416	66	0.07	2.3
F(ab)-12		Ala 60 *Asn* Asp 61 *Pro* Phe 67 *Ile*	CDR-H2; packing of Val 63 and *Ile* 67	501	84	0.08	2.8
MaE11				179	63	0.03	1.0

[a] Murine residues are italicized; residue numbers are according to Kabat et al. (42).
[b] Mean and SD of three soluble receptor assays.
[c] A F(ab)-X/F(ab)-2 ratio >16 means that this variant exhibited no binding even at the highest F(ab) concentrations used.
[d] Changes from F(ab)-1.

from 30 to 50 μg/L based on ELISA. As noted previously (31, 41), the human consensus sequence used allows purification of F(ab) fragments from *E. coli* periplasmic extracts on protein A. F(ab) was always present primarily in the periplasmic extract but detectable at a low level in the media. Mass spectrometry was performed on selected F(ab)s to confirm their m.w.: F(ab)-2, expected M_r 48,303, measured M_r 48,306; F(ab)-9 expected M_r 48,329, measured M_r 48,332; F(ab)-10 expected M_r 48,285, measured M_r 48,286. These values are within the expected error limit of the system (0.01%).

Design of humanized MaE11 antibodies

In contrast to other investigations that have used human sequences closest to the murine Ig of interest (23–27), our humanized antibodies use a human consensus sequence. This consensus sequence consists of a framework based on human VH subgroup III and VLκ subgroup I (42).

First, we constructed F(ab)-1 in which only the six CDRs, as defined by Kabat et al. (42), were grafted onto the human framework—all framework residues were retained as human. This variant is best described as a straight CDR-swap. F(ab)-1 showed no detectable inhibition of IgE binding to its receptor (Table I). Even when one framework residue (H67), subsequently found to be important for maintenance of binding, was replaced with the corresponding murine framework residue, IgE binding was still not restored (F(ab)-1B; Table I). The failure of such "CDR swap" variants to bind their antigens has been reported previously (21, 25).

F(ab)-2 was the first variant based on modeling. In addition to the six murine CDRs, several murine framework residues were incorporated into the human framework (Fig. 1). The definition of CDRs provided by Kabat et al. (42), i.e., based on sequence variability, were used except for CDR-H1 and CDR-H2. CDR-H1 definitions based on sequence variability (42) and on crystallographic studies of antigen-antibody complexes (19) differ significantly (Fig.

1). We therefore redefined CDR-H1 to include both definitions, i.e., residues H26–H35. The definition of CDR-H2 based on sequence variability contains more residues than that based on antibody-antigen crystal structures (Fig. 1). Because none of the crystal structures reported to date show antibody-antigen contacts for antibody residues H60–H65, we used the CDR-H2 definition provided by Chothia et al. (19). Hence, in F(ab)-2 a shorter version of CDR-H2 (residues H50–H58) was used compared with F(ab)-1.

In F(ab)-2 VH, 28 human consensus residues were altered to murine, including only three murine framework residues: *Val* H24, *Ile* H37, and *Phe* H78 (murine residues in all F(ab)s are italicized). In F(ab)-2 VL, 9 human consensus residues were changed to murine, including only one murine framework residue, *Leu* L4. F(ab)-2 is the variant with the minimal number of changes to the human framework which, in our judgment, should be required for maintenance of binding.

An additional 10 variants were constructed to 1) test the effects of buried residues on CDR conformation, 2) determine whether the models had been successful in directing which framework residues should be included in the humanized MaE11, and 3) evaluate the importance of an unusual sequence present in the murine MaE11 (Table I). To test the effects of buried residues on CDR conformation, F(ab)-3 to F(ab)-7 were constructed in which murine residues were changed back to human ones. In F(ab)-3 the buried murine VL residues *Leu* L4 (framework residue) and *Met* L33 (CDR-L1) were exchanged for human sequence Met L4 and Leu L33 to determine their effect on CDR-L1. Chothia et al. (19) have proposed that the residue at position L33 may be important in maintaining proper conformation of CDR-L1. The models suggested that the side chain at L4 might also affect CDR-L1 conformation by its interaction with the side chain at L33. However, binding of F(ab)-3 was only slightly impaired over F(ab)-2. In F(ab)-4 only position L4 was altered and no significant difference in binding compared with F(ab)-2 was found. Together, F(ab)-3 and F(ab)-4 show that, at least for this humanized antibody, the side chains at L4 and L33 have minimal affect on binding and presumably on the conformation of CDR-L1.

The models also suggested that framework residue H24 could affect the conformation of CDR-H1 and framework residue H37 could affect the VL-VH interface (Fig. 2). Substitution of the murine with the human residue at H24 (F(ab)-5) or H37 (F(ab)-7) showed minimal reduction in binding. In contrast, replacing the murine *Phe* at framework position H78 with the human Leu (F(ab)-6) affected a larger reduction in binding. Our models suggest that this side chain is influencing the conformation of CDR-H1 and/or CDR-H2 (Fig. 2). H78 has not previously been considered important in maintaining the conformation of either CDR-H1 or CDR-H2 (19).

In F(ab)-9 to F(ab)-12 human residues were replaced with murine. All four variants exhibited substantial improvement in binding compared with F(ab)-2 (Tables I and II; Fig. 3). In F(ab)-9, which exhibited a fivefold better binding than F(ab)-2, two residues in CDR-H2 (as defined by Kabat et al. (42)) were changed to murine residues: Ala H60 *Asn* and Asp H61 *Pro*. The *Pro* substitution could have altered the CDR-H2 conformation and/or rigidity and *Asn* H60 is anticipated to be buried at the VL-VH interface, possibly interacting with Asp L1 (Fig. 2).

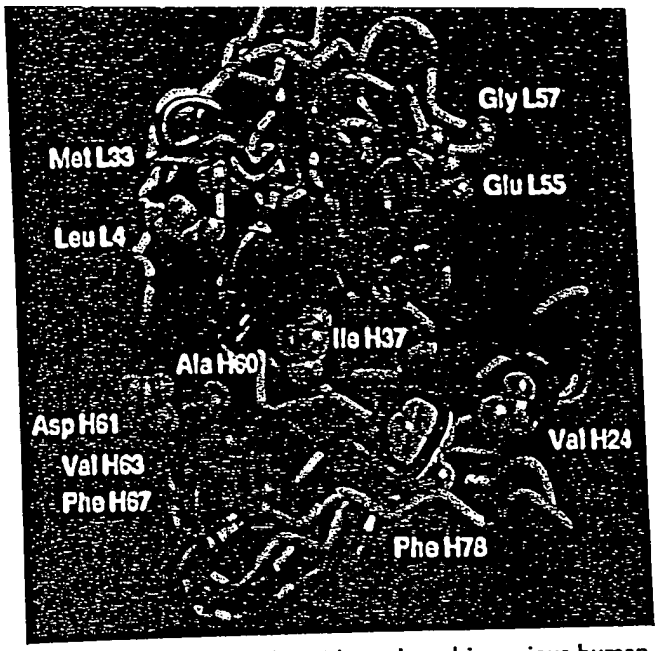

FIGURE 2. Framework residues altered in various humanized MaE11 variants. VL and VH backbone traces are *grey*, VL CDRs are *red*, and VH CDRs are *blue*. Selected framework residue side chain atoms are represented as *spheres*. Leu L4, Met L33, Glu L55, Gly L57, Val H24, Ile H37, and Phe H78 are *yellow*. Ala H60 and Asp H61 are *purple*. Val H63 and Phe H67 are *green*.

F(ab)-10, which also exhibited improved binding relative to F(ab)-2, was a variant in which all buried residues (defined as residues with accessible surface area less than 5% that of the free amino acid) in both VL and VH domains were those of the murine MaE11. In essence F(ab)-10 can be considered as murine MaE11 in which only exposed, non-CDR residues in VL and VH were changed to human sequence. This type of humanized variant was suggested by the recent proposal of Padlan (43). However, the possibility remained that the improved binding exhibited by F(ab)-10 was due to only one or a few residues. For example, F(ab)-10 contains Ala H60 *Asn* that, based on F(ab)-9 (Table I), could alone account for much of the improved binding of F(ab)-10.

Two additional variants were evaluated to test this possibility. For these two variants, instead of using F(ab)-2 as the basis, we used F(ab)-9 because this variant showed a fivefold improved binding (Table I). According to the models, the side chains at H63 and H67 could affect the con-

Table II
Humanized MaE11 IgG1 variants

Variant[a]	Concentration at 50% inh. (ng/ml)		Variant X	Variant X
	Mean	SD[b]	IgG1–2	MaE11
IgG1–2	7569	1042	1.0	16.9
IgG1–9	3493	1264	0.46	7.8
IgG1–10	1118	172	0.15	2.5
IgG1–12	1449	226	0.19	3.2
MaE11	449	53	0.06	1.0

[a] IgG1–2 represents full-length IgG1 molecule, variant 2.
[b] Mean and SD of five soluble receptor assays.

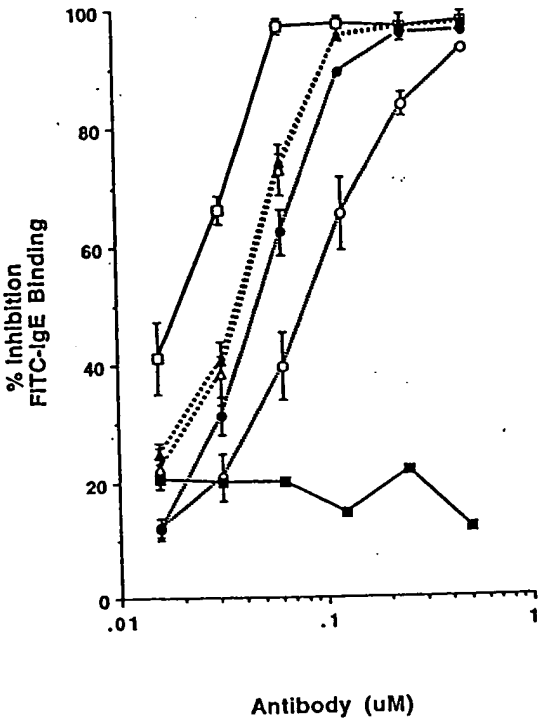

FIGURE 3. Murine MaE11 and humanized F(ab)s block FITC-IgE binding to CHO 3D10 cells expressing FcεRI α-chain. Percentage of inhibition by murine mAb MaE11 (□), F(ab)-2 (○), F(ab)-9 (●), F(ab)-11 (△), F(ab)-12 (▲), and the negative control humanized mAb 4D5 (21) (■) measured by FACS. Data points are the average of three experiments, except for mAb 4D5 (single experiment).

formation of CDR-H2 (Fig. 2). H63 is part of CDR-H2, as defined by Kabat et al. (42), but not as defined by Chothia et al. (19), whereas H67 is defined as a framework residue under both CDR definitions. In F(ab)-11 H63 and H67 were the murine residues *Leu* and *Ile*, respectively. In F(ab)-12 only H67 was changed to the murine *Ile* (H63 remained as the human Val). In both the soluble receptor and cell-based assays these variants exhibited binding that was at least as good as F(ab)-10 and better than their parent, F(ab)-9- (Tables I and II; Fig. 3). This suggests that the improved binding of F(ab)-10 was not due to repacking of the VH domain interior with murine residues, but was due to the F(ab)-8 was constructed to examine an unusual sequence in MaE11 VL: *Gly* L55-*Ser* L56-*Glu* L57. In both murine and human VLκ sequences L57 is conserved as Gly and in human VLκ subgroup I Glu predominates at L55 (42). F(ab)-2 used the human sequence at these two positions and F(ab)-8 the murine. This had no effect on binding to IgE (Table I).

Once we determined which F(ab) variant provided binding closest to murine MaE11, we generated full-length IgG1 molecules. The binding of these molecules relative to variant 2 or murine MaE11 (Table II) was comparable to the relative binding exhibited by the F(ab) fragments (Table I). Note, however, that for variants 2 and 10 the ratio of the F(ab) relative to murine MaE11 was twofold higher than for the corresponding IgG1 relative to murine MaE11. This may be due to error in ascertaining the protein concentration or differences in the avidity of the IgG1 form of these variants compared with the other variants.

Binding of MaE11 to IgE-loaded FcεRI

Murine MaE11 prevents binding of free IgE to FcεRI on mast cells but does not trigger histamine release by binding to IgE-loaded FcεRI (unpublished data). As shown in Figure 4, both murine MaE11 and humanized variant 12, as well as the negative isotype control antibody MOPC21 and negative isotype control humanized 4D5 (21), did not bind IgE-loaded FcεRI on CHO 3D10 cells. In contrast, the murine MaE1 antibody, which binds to IgE but does not prevent IgE binding to FcεRI, bound to the IgE-loaded FcεRI. Unlike the human IgG1 control (humanized 4D5), the murine IgG1 isotype (as represented by MOPC21) exhibits a nonspecific background binding of approximately 10% on these cells. MaE11 did not give staining above the MOPC21 control and humanized variant 12 did not give staining above the humanized 4D5 control (Fig. 4). The failure to detect binding of MaE11 or humanized variant 12 to IgE on 3D10 cells was not due to displacement of receptor-bound IgE by the antibodies. Using a polyclonal anti-IgE reagent, the amount of IgE detected on these cells after a 1-h incubation with MaE11 or humanized variant 12 was equivalent to the level measured in the absence of antibodies (data not shown).

CDR residues important in IgE binding

The sequence of the MaE11 CDRs shows a preponderance of charged side chains (Fig. 1). CDR-L1 contains three Asp residues, whereas CDR-L3 possesses His, Glu, and Asp; in CDR-H3 there are three His residues. The models of murine and humanized MaE11 pointed to the spatial proximity of all of these charged residues (Fig. 5). In contrast, the lone Asp H54 in CDR-H2 is spatially separated from the other charged residues. Though we did not attempt an exhaustive

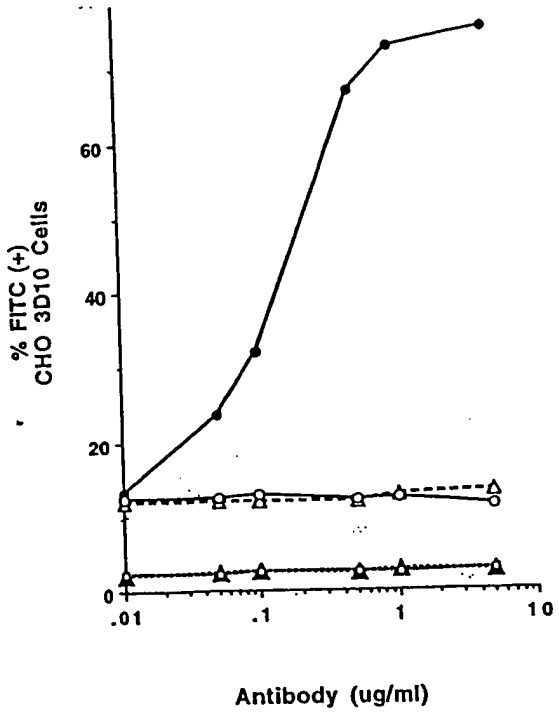

FIGURE 4. Murine MaE11 and humanized variant 12 do not bind to IgE-loaded CHO 3D10 cells expressing FcεRI α-chain. Percentage of binding by murine mAb MaE11 (○), humanized mAb variant 12 (▲), positive control murine mAb MaE1 (●), negative control antibody murine MOPC21 (△), and the negative control humanized mAb 4D5 (21) (□) measured by FACS. On an arithmetic/linear scale, mean channel fluorescence values at 10 μg/ml were MOPC21 7.3, MaE1 32.1, MaE11 6.4, hu4D5 4.7, and huMaE11 4.6. All three murine mAbs were murine isotype IgG1, and both humanized mAbs were human isotype IgG1. Data points are the average of three experiments.

analysis of all CDR residues, we did focus on these charged residues, generating variants in which one or more charged residues were replaced with Ala. In CDR-L1 alteration of one of the three Asp residues, Asp L30, effectively abolished binding to IgE (F(ab)-16; Table III), whereas the other two had minimal effect (F(ab)-14, F(ab)-15). Simultaneous alteration of GluL93Ala and AspL94Ala in CDR-L3 (F(ab)-17; Table III) also reduced binding, although much less than for AspL30Ala. Individually substituting the three His residues in CDR-H3 with Ala resulted in either slightly improved binding (F(ab)-21) or a threefold reduction in binding (F(ab)-20 and F(ab)-22). However, simultaneous alteration of all three His residues abolished binding (F(ab)-19). Although we cannot discern whether these charged residues are involved in direct binding to IgE or provide some conformational stability to their respective CDRs, variants F(ab)-13 to F(ab)-22 show that CDR-L1 and CDR-H3 are important determinants in IgE binding.

We have described herein the humanization of the murine antibody MaE11, which is targeted against human IgE. Design of a functional antibody was dependent on substitution of several murine framework residues into the human framework. In addition, we mapped the charged CDR residues and found some of them to be important in the antibody-IgE interaction.

In agreement with previous studies (21, 23–25), variants 1 to 12 show that framework residues can have a significant effect on antibody function. This is underscored when considering F(ab)-1, which is a straight CDR swap in which only the six CDRs were transplanted onto the human framework. No consideration was given to altering framework residues, all of which were retained as human. Table I shows that, even at high concentrations, F(ab)-1 did not inhibit IgE binding to its receptor. Inclusion of murine framework residue *Phe*H78 (F(ab)-1B) still did not restore inhibitory activity.

At least two possibilities might account for the lack of binding of F(ab)-1 to IgE. The first involves residues L4 and L33. In F(ab)-2 these residues were *Leu* L4 and *Met* L33; in F(ab)-1 they were Met*L4* and *Met* L33. Conceivably, the Met *L4-Met* L33 combination in F(ab)-1 might disturb the conformation of CDR-L1. However, we isolated the Met *L4-Met* L33 combination from the other changes in F(ab)-1 (F(ab)-4; Table I) and this variant showed minimal affect on binding (Table I).

A second possible problem in F(ab)-1 involves CDR-H2. The buried hydrophobic side chains at positions H63 and H67 could affect the conformation of CDR-H2 (Fig. 2). Our variants contain four combinations at positions H63 and H67: *Leu* and *Ile* (murine MaE11 and F(ab)-11), Val and Phe (F(ab)-2), *Leu* and Phe (F(ab)-1), and Val and *Ile* (F(ab)-12). The binding of these four variants (Table I) indicates that the important residue is H67, which must be the murine *Ile* to provide affinity comparable to murine MaE11. This residue was the human Phe in F(ab)-1.

Of the 12 residues in F(ab)-1 retained as human (compared with F(ab)-2), 8 were separately changed to murine in other variants. Three had no affect on binding: L4 (F(ab)-4), L55, and L57 (F(ab)-8). Two residues, H60 and H61 (F(ab)-9), improved binding, whereas three reduced binding: H24 (F(ab)-5), H37 (F(ab)-7), and H78 (F(ab)-6). We cannot therefore assign any individual framework residue in F(ab)-1 as solely responsible for its lack of binding, and the possibility remains that the combination of several incorrect framework residues (e.g., at positions H24, H37, H67, and H78) could act in concert to destroy binding affinity.

We also tested the recent proposal by Padlan (43) that the immunogenicity of a murine antibody could be reduced by changing only exposed framework residues, thus making it

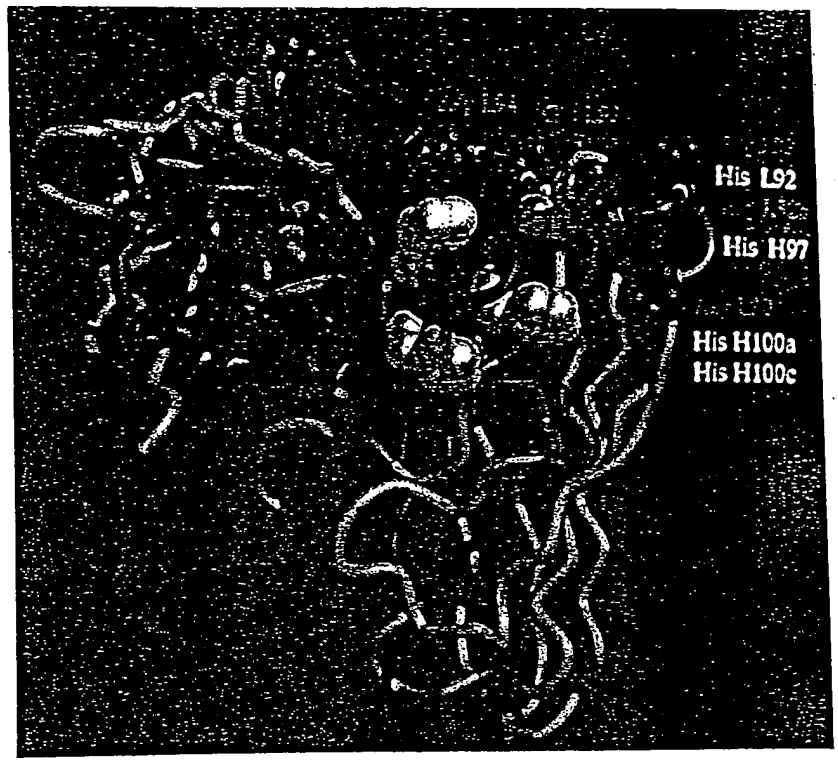

FIGURE 5. Charged CDR side chains in MaE11. VL and VH backbone traces are *grey*, VL CDRs are *red*, and VH CDRs are *blue*. Charged CDR side chain atoms are represented as *spheres*. Acidic side chains are *green* and basic side chains are *yellow*.

Table III
Humanized MaE11 F(ab) CDR residue variants

Variant	Changes from F(ab)-2[a]		Purpose	Concentration at 50% inh. (ng/ml)		F(ab)-X
	VL	VH		Mean	SD[b]	F(ab)-2
F(ab)-2				6083	1279	1.0
F(ab)-13	*Asp* 29a Ala *Asp* 29c Ala *Asp* 30 Ala		CDR-L1 binding	>100,000		>16.0[c]
F(ab)-14	*Asp* 29a Ala		CDR-L1 binding	3452	183	0.57
F(ab)-15	*Asp* 29c Ala		CDR-L1 binding	6384	367	1.0
F(ab)-16	*Asp* 30 Ala		CDR-L1 binding	>100,000		>16.0
F(ab)-17	*Glu* 93 Ala *Asp* 94 Ala		CDR-L3 binding	17,456	7115	2.9
F(ab)-18		*Asp* 54 Ala	CDR-H2 binding	2066	174	0.34
F(ab)-19		*His* 97 Ala *His* 100a Ala *His* 100c Ala	CDR-H3 binding	>100,000		>16.0
F(ab)-20		*His* 97 Ala	CDR-H3 binding	19,427	8360	3.2
F(ab)-21		*His* 100a Ala	CDR-H3 binding	2713	174	0.45
F(ab)-22		*His* 100c Ala	CDR-H3 binding	15,846	8128	2.6

[a] Murine residues are italicized; residue numbering according to Kabat et al. (42) with insertions denoted by lowercase suffix.
[b] Mean and SD of three soluble receptor assays.
[c] A F(ab)-X/F(ab)-2 ratio >16 means that this variant exhibited no binding even at the highest F(ab) concentrations used.

easier to retain antigen binding (compared with transplanting CDRs onto a human framework). F(ab)-10 was constructed by repacking the hydrophobic interior of both the VL and VH domains with murine residues; this variant can be considered as murine MaE11 in which only exposed framework residues in VL and VH were changed to human sequence. Though F(ab)-10 exhibited binding close to that of the murine MaE11, we found that changing a single amino acid in the VH domain (H67) from human to murine could effect the same improved binding (F(ab)-12, IgG1–12; Tables I and II; Fig. 3).

The CDRs of MaE11 contain several charged residues (Figs. 1 and 5). A number of these are involved in binding to the IgE (Table III), although we cannot discern whether this is through direct contact with the IgE or through stabilization of CDR conformation. The concentration of

charges in the CDRs, especially in [illegible] suggests that the MaE11 epitope on IgE has a concentration of charged residues.

Humanized MaE11 was designed using a consensus human framework derived from amino acid sequences of human VLκ subgroup I and human VH subgroup III. For the humanized variant exhibiting binding comparable to murine MaE11—F(ab)-12—only five human framework residues were replaced with murine (L4, H24, H37, H67, and H78). Four of these were determined by initial use of molecular models. The fifth, H67, as well as CDR-H2 residues H60 and H61, were included by using the molecular models in an effort to improve the binding of the initial variant F(ab)-2. Other investigators have used a human antibody sequence closest to the murine antibody of interest as a basis for humanization (23–27). The success of this humanization, in conjunction with the success of previous humanizations using the same human framework (21, 22), shows that a single framework can indeed function with various murine CDRs. This assumes, however, that the important murine framework residues are included in the humanized version.

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